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Published in:
FEBS Letters

DOI:
[10.1016/j.febslet.2005.09.065](https://doi.org/10.1016/j.febslet.2005.09.065)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Dudkina, N. V., Heinemeyer, J., Keegstra, W., Boekema, E. J., & Braun, H-P. (2005). Structure of dimeric ATP synthase from mitochondria: An angular association of monomers induces the strong curvature of the inner membrane. *FEBS Letters*, 579(25), 5769 - 5772. <https://doi.org/10.1016/j.febslet.2005.09.065>

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Structure of dimeric ATP synthase from mitochondria: An angular association of monomers induces the strong curvature of the inner membrane

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Received 20 September 2005; accepted 27 September 2005

Available online 6 October 2005

Edited by Richard Cogdell

Abstract Respiration in all cells depends upon synthesis of ATP by the ATP synthase complex, a rotary motor enzyme. The structure of the catalytic moiety of ATP synthase, the so-called F₁ headpiece, is well established. F₁ is connected to the membrane-bound and ion translocating F₀ subcomplex by a central stalk. A peripheral stalk, or stator, prevents futile rotation of the headpiece during catalysis. Although the enzyme functions as a monomer, several lines of evidence have recently suggested that monomeric ATP synthase complexes might interact to form a dimeric supercomplex in mitochondria. However, due to its fragility, the structure of ATP synthase dimers has so far not been precisely defined for any organism. Here we report the purification of a stable dimeric ATP synthase supercomplex, using mitochondria of the alga *Polytomella*. Structural analysis by electron microscopy and single particle analysis revealed that dimer formation is based on specific interaction of the F₀ parts, not the F₁ headpieces which are not at all in close proximity. Remarkably, the angle between the two F₀ part is about 70°, which induces a strong local bending of the membrane. Hence, the function of ATP synthase dimerisation is to control the unique architecture of the mitochondrial inner membrane.

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Keywords: ATP synthase; Dimer; Electron microscopy; *Polytomella*

1. Introduction

Mitochondrial F₁F₀ ATP synthase or ATPase is a complex of about 600 kDa formed by 15–18 distinct subunits. Five of these subunits (α , β , γ , δ , ϵ) constitute the F₁ headpiece, which has been resolved by X-ray crystallography [1,2]. In contrast, the F₀ part is structurally less well defined. It is composed of a ring of 9–12 copies of the lipoprotein “subunit c” attached to two larger subunits termed subunits a and b. A number of additional small subunits form part of F₀ and the peripheral stalk. In yeast, three of them, termed subunits e, g and k, only occur in a dimeric 1200 kDa ATP synthase supercomplex, which was described recently [3]. Yeast cells deficient in the di-

mer-specific subunits e or g lack dimeric ATP synthase [4,5]. Recent data also point to an involvement of subunit b in dimer formation [6]. Similar dimeric and oligomeric ATP synthase supercomplexes were also found in bovine heart [7], *Arabidopsis* [8] and *Chlamydomonas* [9]. However, so far knowledge on dimeric ATP synthase is limited due to the lack of structural data.

Dimeric ATP synthase from *Chlamydomonas* was found to be especially stable [9–11]. Compared to ATP synthase dimers from other organisms it includes an additional 60 kDa protein designated “mitochondrial ATP synthase associated protein” (MASAP), which is speculated to be involved in dimer formation [9]. A very similar ATP synthase supercomplex is present in the closely related non-green alga *Polytomella* [12]. Here we report purification and structural characterization of the ATP synthase supercomplex from *Polytomella*.

2. Materials and methods

2.1. Cultivation of *Polytomella*

Polytomella spp. (198.80, E.G. Pringsheim) was obtained from the “Sammlung von Algenkulturen der Universität Göttingen (SAG)” (<http://www.epsag.uni-goettingen.de/html/sag.html>). Cells were cultivated for 4–5 days in 2.5-liter culture flasks including 200 ml medium [0.2% (w/v) sodium acetate, 0.1% (w/v) yeast extract and 0.1% (w/v) tryptone] at 25 °C in the dark without shaking.

2.2. Preparation of mitochondria

For mitochondrial isolations, cells were sedimented by centrifugation at 2000 × g for 10 min, washed twice in 20 mM sodium phosphate buffer, pH 7.4 and finally re-suspended in 0.32 M sucrose, 4 mM EDTA, 20 mM Tris–HCl, pH 7.4. Disruption of cells was carried out using a “Dounce Homogenisator” (10 strokes). Organelles were enriched by differential centrifugation for 8 min at 1000 × g (mitochondria in supernatant), 8 min at 1000 × g (mitochondria in supernatant) and 15 min at 10000 × g (mitochondria in pellet). The resulting crude mitochondrial fraction was re-suspended in gradient buffer (0.4 M mannitol/0.1% BSA, 1 mM EGTA, 0.2 mM PMSF, 10 mM KH₂PO₄, pH 7.2) and organelles were purified by Percoll density gradient centrifugation (14%, 22%, 45% Percoll in gradient buffer) at 70000 × g for 45 min. Finally, *Polytomella* mitochondria were washed twice by centrifugation at 15000 × g at 10 min and re-suspended in gradient buffer at a protein concentration of 10 mg/ml.

2.3. Purification of dimeric ATP synthase

Isolated mitochondria were treated with solubilization buffer (30 mM HEPES, 150 mM K-acetate, 10% glycerol, 5% digitonin, pH 7.4) and solubilized protein complexes were separated by sucrose

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gradient ultracentrifugation (gradients of 0.3–1.5 M sucrose, 15 mM Tris base, pH 7.0, 20 mM KCl, 0.2% digitonin; centrifugation for 17 h at $150000 \times g/4^\circ\text{C}$). Afterwards, fractions were removed from the gradient from bottom to top. 1D Blue-native PAGE and 2D Blue-native/SDS-PAGE was carried out as described by Schagger [13].

2.4. Electron microscopy and single particle analysis

Electron microscopy and single particle analyses including alignments of projections with multi-reference and non-reference procedures, multivariate statistical analysis and classification, was carried out as outlined by Dudkina et al. [14].

3. Results and discussion

3.1. Purification of ATP synthase dimers

Total mitochondrial membranes from *Polytomella* were solubilized by digitonin and protein complexes were separated by sucrose gradient ultracentrifugation (Fig. 1). A 1D Blue-native PAGE was carried out to monitor the protein complex composition of the fractions of the sucrose gradient (Fig. 1, left gel). Identification of the protein complexes was based on their subunit composition by a parallel 2D Blue-native/SDS-PAGE of total mitochondrial protein from *Polytomella* (Fig. 1, right gel). On the 2D gels, dimeric ATP synthase migrates well above complex I and is resolved into the known subunits, including MASAP [9]. Monomeric ATP synthase was not detectable. The respiratory chain complexes I, III₂ and IV are present as monomeric complexes, but partially also form large supermolecular structures, which run on the top of the 2D gel. The highest concentration of dimeric ATP synthase was in fraction 5 of the sucrose gradient and this fraction

was used for a structural characterization by electron microscopy and single particle analysis [14].

3.2. Structural analysis by single particle electron microscopy

Projection maps were obtained after classification of large numbers of particles. The best classes have a resolution of about 17 Å, according to the Fourier-ring correlation criterion (see [14]). They show how the interaction of the F₁F₀ ATP synthase monomers occurs within the dimer (Fig. 2(a)). The projection map indicates that most if not all of the interaction is between the membrane-bound F₀ parts. Surprisingly, the F₁ headpieces are not at all in close proximity and the stator structures are much more substantial than in any other monomeric F- or V-ATPase [15], likely because of the special dimer-specific subunits such as the MASAP subunit of 60 kDa. The most remarkable feature is the angle of about 70° between the F₀ parts, which implies a strong local bending of the inner mitochondrial or cristae membrane around the dimers. This angle was found for the vast majority of the dimers and is even maintained in those particles in which one of the headpieces was dissected (Fig. 2(b) and (c)). The angle of 70° between the ATP synthase monomers deviates by up to $\pm 5^\circ$ in a small number of dimers only (results not shown), indicating a specific type of interaction.

4. Discussion

We have shown for the first time the projection structure of an ATP synthase dimer in which the F₁ and F₀ parts are well

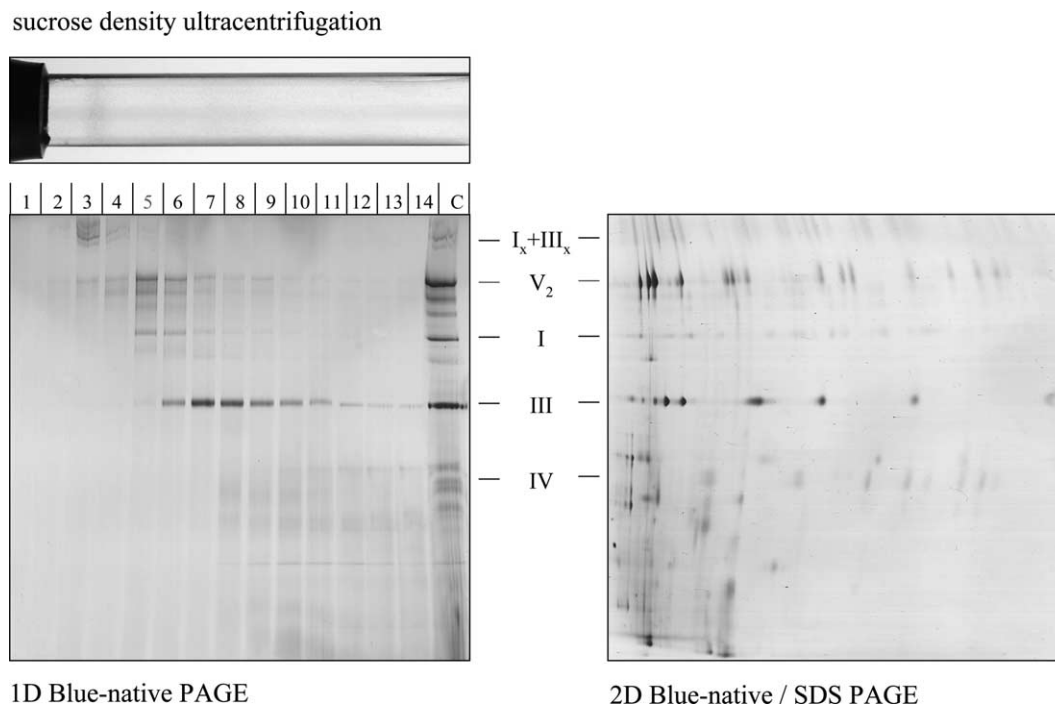


Fig. 1. Purification of dimeric ATP synthase of *Polytomella* by sucrose gradient ultracentrifugation. *Top*. Separation of mitochondrial protein complexes by ultracentrifugation. *Left gel*. 1D Blue-native PAGE of the fractions of the gradient. Numbers on top of the gel refer to the fractions of the sucrose gradient (from bottom to top as indicated); C: total mitochondrial protein. *Right gel*. 2D Blue-native/SDS-PAGE of total mitochondrial protein of *Polytomella*. Identities of protein complexes are indicated between the two gels: IV, complex IV; III₂, dimeric complex III; I, complex I; V₂, dimeric complex V; I_x + III_x, supercomplex including complexes I and III. Fraction 5 was selected for electron microscopy and single particle analysis.

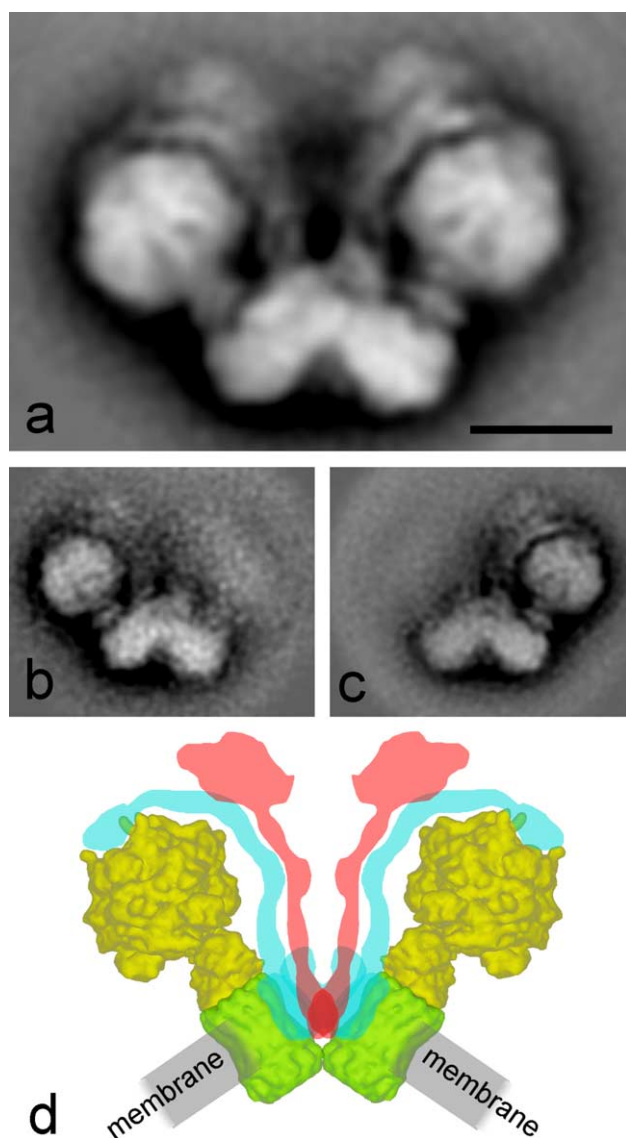


Fig. 2. Electron microscopy analysis and interpretation. (a) Projection map of dimeric ATPase. The average 2D projection is the sum of a homogeneous subset of 3000 projections out of 40 000 projections found by statistical analysis and classification. Bar is 10 nm. (b,c) Dimeric ATP synthase fragments lacking one specific F_1 headpiece. The class-sums are comprised of about 500 projections each. (d) Interpretation. Yellow, F_1 headpiece and central stalk; green, c-subunit rotor component of F_0 ; blue, peripheral stalk and red, dimer-specific components, not present in monomeric ATPase.

resolved, including flanking peripheral stalks or stators. The projection maps of the complete dimers and the fragments missing one of the F_1 parts clearly indicate that the interaction between the monomers is within their F_0 parts. In parallel to our current investigation another dimeric ATP synthase was studied by Minnauro-Sanmiguel et al. [16]. Analysis of the dimer from beef heart mitochondria indicates that the monomers also make an angle. In comparison to the dimer from *Polytomella* the angle is substantially smaller and about 40° and the F_1 parts seem to be (almost) in contact. Although the authors present a scheme in which two peripheral stalks or stators should be in between the two F_1 parts the processed images do not show such peripheral stalks. Thus it

is likely that most of the dimers do not contain the peripheral stalk anymore. On the other hand it could be possible that this small and thin stator is difficult to see because of overlap in projection with other subunits or a limited resolution. However, the loss of a stator often co-insides with absence of the OSCP subunit, which is located at the top of the F_1 part. This position has been convincingly shown by immuno-labelling of the homologous δ subunit in *Escherichia coli* [17]. This investigation indicates that the presence of the δ subunit causes the F_1 part to end in a sharp tip, like OSCP in our images and in contrast to the blunt tip as presented in [16]. We therefore are not convinced about the intactness of the monomers in the ATP synthase dimer of beef. In contrast, the peripheral stalks are clearly visible in the *Polytomella* dimer.

Until now there is no emerging role for the dimeric ATPase, although it was speculated that the ATP synthase supercomplex formation might optimize energy transduction [3]. Alternatively, it has been proposed that dimers could be involved in the control of the biogenesis of the inner mitochondrial membrane [4,5]. The authors of the latter studies suggested that there should be a link between ATP synthase dimerization and the cristae morphology, because deletion of dimer-specific subunits changes the overall morphology of the membrane foldings. Yeast mutants unable to form dimeric ATP synthase comprise mitochondria with drastically changed morphology, which lack the characteristic highly folded inner membrane architecture, the cristae. Instead the membranes consist of atypical “onion-like” structures. A similar mitochondrial morphology was observed in yeast cells containing in vivo cross-linked F_1 -headpieces [18], providing further evidence for a role of dimerization of ATP synthase for cristae formation.

Our data provide a direct clue for the role of dimerization of ATP synthase monomers. It is proposed to be the driving force for cristae formation and overall mitochondrial morphology because the unique way of the out of plane association of the F_0 membrane domains will force a strong local curvature of the membrane (Fig. 2(d)). It should be realized that the bulk of the ATP synthase complexes is not part of a rather flat inner mitochondrial membrane, but present within curved invaginations known as cristae lamellae and tubules [19]. For tubular membranes, the diameter is often in the range 24–32 nm, if the width of the bilayer is included [19]. If the bent membrane in the region of the dimers is regarded as a 70° arc section of radius 16 nm, this configuration could by extrapolation induce a tubule with a diameter of about 25 nm. Such a diameter would fit the observed cristae dimensions nicely. Likely the ATP synthase dimers would associate in specific oligomers with the other respiratory chain supercomplexes such as the supercomplex formed by monomeric complex I and dimeric complex III [14] in between. Indeed oligomeric ATP synthase structures were previously described by rapid-freeze deep-etch EM [19,20] and by Blue-native PAGE [4,5,21,22]. We propose that dimeric ATP synthase supercomplexes represent basic building blocks of ATP synthase oligomers and that formation of these structures is the driving force for cristae formation and overall mitochondrial morphology. It should finally be remarked that a spectacular surface extension of the inner membrane is realized by multiple foldings of this membrane. It has for instance been estimated that the surface of the inner mitochondrial membrane in an average human would be around 14 000 m² [23].

Acknowledgements: H.P.B. acknowledges a grant of the Deutsche Forschungsgemeinschaft (Br1829-7/1) and E.J.B. a grant of the Dutch science foundation NWO-CW.

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